PRODUCTION OF PRAVASTATIN BY FILAMENTOUS FUNGI ISOLATED FROM SOIL

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ABSTRACT

Pravastatin is a clinically useful cholesterol-lowering agent, selectively inhibiting 3-hydroxy-3-methylglutaryl-coenzyme A reductase, the regulatory enzyme in cholesterol biosynthesis. Currently, industrial production of this statin is based on a two-step fermentation process: the initial production of compactin and its subsequent biotransformation to pravastatin. The development of a one-step fermentation process using pravastatin-producing microfungi may be a commercially attractive approach. To facilitate this, isolation of novel fungal strains from different natural sources and their screening for pravastatin production is required. Soil being a reservoir for a wide variety of filamentous fungi has been recognized for a long time. In this study, 54 fungal cultures were obtained from soil samples collected in Pahang State (Malaysia). Isolates were cultivated in submerged fermentation and tested for their ability to produce pravastatin using high-performance liquid chromatography. Five selected pravastatin producers were identified to species level using cultural and morphological characteristics, physiological and biochemical tests, and molecular techniques. Screening of the important variables affecting pravastatin production by the best of these producers was initially carried out using 2^{7-3} fractional factorial design and these selected variables were then optimized using rotatable central composite design. Kinetic studies of substrate uptake, fungal growth and pravastatin production in shake flask culture under optimized conditions were also conducted. Among 25 *Penicillium* isolates that were capable of producing pravastatin directly by fermentation, only five (ESF2M, ESF19M, ESF20P, ESF21P, and ESF26P) did so in relatively high concentrations, with *Penicillium* sp. ESF21P being the most active pravastatin producer, achieving a concentration of 196.83 mg/L. Fungal identification methods used in this study confirmed that the isolates *Penicillium* sp. ESF2M and ESF19M are referable to *Penicillium citrinum*, *Penicillium* sp. ESF20P and ESF26P were most closely related to *Penicillium janthinellum*, and *Penicillium* sp. ESF21P showed the highest homology with *Eupenicillium brefeldianum*. All sequence data from this study have been deposited in the GenBank database. A maximum concentration of 234.36 mg/L of pravastatin was produced by *E. brefeldianum* ESF21P under the optimized conditions suggested by the Design-Expert 6.0.8 software. Pravastatin fermentation using this fungus showed the typical kinetics of a secondary metabolite, with maximum yield obtained after about 288 h of fermentation.

ABSTRAK

Pravastatin merupakan satu agen perendah-kolesterol yang merencat 3-hidroksi-3-methilglutaril-koenzim A reductase secara klinikal. 3-hidroksi-3-methilglutaril-koenzim A reductase merupakan enzim pengatur dalam biosintesis kolesterol. Sehingga kini penghasilan statin adalah berasaskan satu proses fermentasi dua-langkah: penghasilan kompaktin pada peringkat permulaan dan diikuti dengan biotransformasinya kepada pravastatin. Pembangunan satu proses fermentasi satu-langkah menggunakan mikrofungi penghasil-pravastatin adalah satu pendekatan komersil yang menarik. Pemencilan strain fungi novel daripada sumber semulajadi dan penyaringannya adalah perlu untuk penghasilan pravastatin. Sejak dahulu lagi tanah merupakan sumber terbaik untuk pelbagai fungi berfilamen. Dalam kajian ini, 54 kultur fungi didapati daripada sampel-sampel tanah yang dikutip dalam negeri Pahang (Malaysia). Pencilan-pencilan ini dibiakkan dalam fermentasi tenggelam dan diuji kemampuannya untuk menghasilkan pravastatin menggunakan kromatografi cecair berprestasi tinggi. Lima spesis fungi penghasil pravastatin telah dikenal pasti daripada pencirian kultur dan morfologi, ujian-ujian fisiologi dan biokimia, dan teknik-teknik molekul. Penyaringan pembolehubah-pembolehubah penting yang mempengaruhi penghasilan pravastatin oleh penghasil terbaik telah dilakukan pada peringkat awal dengan menggunakan rekabentuk faktorial 2^{7-3}. Kemudian aras pembolehubah-pembolehubah yang dipilih melalui saringan ini dioptimumkan dengan menggunakan rekabentuk boleh-putar komposit berpusat. Kajian kinetik penggunaan substrat, tumbesaran fungi dan penghasilan pravastatin dalam kultur kelalang goncang pada keadaan optimum telah juga dilakukan. Daripada 25 pencilan *Penicillium* yang boleh menghasilkan pravastatin, hanya lima (ESF2M, ESF19M, ESF20P, ESF21P, dan ESF26P) boleh menghasilkannya dalam kepekatan yang relatif tinggi. Didapati *Penicillium* sp. ESF21P merupakan penghasil pravastatin yang paling aktif, mencapai kepekatan 196.83 mg/L. Kaedah-kaedah pengenalpastian fungi yang digunakan dalam kajian ini telah mengesahkan bahawa pencilan-pencilan *Penicillium* sp. ESF2M dan ESF19M boleh dirujuk kepada *Penicillium citrinum*. *Penicillium* sp. ESF20P dan ESF26P adalah paling berkait rapat dengan *Penicillium janthinellum*, dan *Penicillium* sp. ESF21P menunjukkan homologi tertinggi dengan *Eupenicillium brefeldianum*. Semua data jujukan daripada kajian ini telah telah disimpan di pengkalan data GenBank. Pada keadaan optimum yang dicadangkan oleh perisian Design-Expert 6.0.8, *E. brefeldianum* ESF21P telah mencatatkan kepekatan maksimum pravastatin (234.36 mg/L). Fermentasi pravastatin menggunakan fungi ini menghasilkan nilai kinetik tipikal bagi metabolit sekunder di mana hasil maksimum dicapai selepas kira-kira 288 jam fermentasi.

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LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
AR	Agitation rate
bp	Base pair
CCD	Central composite design
CD	Colony diameter
cm	Centimeter
CoA	Coenzyme A
COC	Colony obverse color
CRC	Colony reverse color
CREA agar	Creatine-Sucrose Agar
$CuSO_4 \cdot 5H_2O$	Copper (II) sulphate pentahydrate
CYA	Czapek Yeast Autolysate Agar
CYAS	Czapek Yeast Autolysate with 5% NaCl
CZ	Czapek-Dox Agar
d	Day
2D	Two-dimensional
3D	Three-dimensional
DAD	Diode array detection
DCW	Dry cell weight
DIST	Distance

DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
$FeSO_4 \cdot 7H_2O$	Ferrous sulfate heptahydrate
FF	Filamentous fungi
FFD	Fractional factorial design
2FI	2- Factor interaction model
3FI	3-Factor interaction model
FT	Fermentation time
g	Gram
GC	Gas chromatography
Glu	Glucose
Gly	Glycerol
h	Hour
ha	Hectare
HCl	Hydrochloric acid
HDL	High-density lipoprotein
HMG	3-Hydroxy-3-methylglutaryl
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
HPLC	High- performance liquid chromatography
H_3PO_4	Phosphoric acid
ID	Internal diameter
IFO	Institute for Fermentation

ITS 1	Internal transcribed spacer 1
ITS 2	Internal transcribed spacer 2
IUPAC	International Union of Pure and Applied Chemistry
IV	Inoculum volume
kb	Kilobase
KCl	Potassium chloride
kg	Kilogram
KH_2PO_4	Potassium dihydrogen phosphate
$K_2HPO_4 \cdot 3H_2O$	Dipotassium hydrogen phosphate
L	Litre
LC	Liquid chromatography
LDL	Low-density lipoprotein
m	Metre
mAU	Milliabsorbance unit
MEA	Malt Extract Agar
mg	Milligram
$MgCl_2$	Magnesium chloride
$MgSO_4 \cdot 7H_2O$	Magnesium sulfate heptahydrate
min	Minute
mL	Millilitre
ML-236B	Mevastatin
mm	Millimetre

MS	Mass spectrometry
N	Normality
NaCl	Sodium chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
$NaNO_2$	Sodium nitrite
$NaNO_3$	Sodium nitrate
NaOH	Sodium hydroxide
Na_2SO_4	Sodium sulphate
ND	Not detected
ng	Nanogram
NHMS	National Health and Morbidity Survey
nm	Nanometre
NO_2 agar	Nitrite-Sucrose Agar
NTG	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
OD	Optical density
PCR	Polymerase Chain Reaction
PDA	Potato-Dextrose Agar
pH	Hydrogen ion concentration
PROB	Probability
PTFE	Polytetrafluoroethylene
rDNA	Ribosomal deoxyribonucleic acid
RID	Refractive index detector